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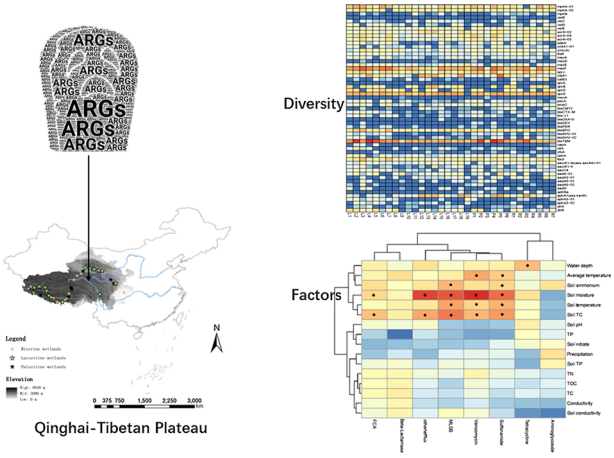
Bacterial community and climate change implication affected the diversity and abundance of antibiotic resistance genes in wetlands on the Qinghai-Tibetan Plateau

Yuyi Yang[a,b], Guihua Liu[a], Chen Ye[a], Wenzhi Liu[a,*]

[a] Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan, 430074, China
[b] School of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland, UK

GRAPHICAL ABSTRACT



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ABSTRACT

Antibiotic resistance genes (ARGs) have been identified as emerging pollutants in the environment. However, little information is available for ARGs in natural wetlands at high altitude. In this study, we investigated 32 wetlands across the Qinghai-Tibetan Plateau, with the variation of wetland types, altitude, and environmental factors, to assess the determinant factor of ARGs in this area. ARGs were detected in all the wetlands, ranged from 1.80 × 105 to 1.35 × 107 copies per gram of soils. The ARGs in wetland soils were diverse and abundant, and varied from each site, but the spatial geographical distance did not influence the ARG profile. The mobile genetic elements in wetlands ranged from 3.13 × 103 to 1.02 × 106 copies per gram of soil, indicating the low abundance of mobile genetic elements suggests a lower transfer rate of ARGs between bacteria in the Plateau. Bacterial community composition was the main driver in shaping the ARG diversity and geographic distribution. Soil moisture and temperature were positively correlated with ARG abundance in this region. These results not only provide a better understanding of the background levels of ARGs in the Qinghai-Tibetan Plateau, but also shed light on the influence of climate change and increased human activities on the distribution of ARGs.

* Corresponding author at: Wuhan Botanical Garden, Chinese Academy of Sciences, Lumo Road No.1, Wuchang District, Wuhan, China.
E-mail address: liuwz@wbgcas.cn (W. Liu).

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1. Introduction

As the Earth's highest elevated plateau, the Qinghai-Tibet Plateau, often referred as the Third Pole [1], is a unique geographical region [2]. This region, with its complex interactions of atmospheric, cryospheric, hydrological, geographic, and environmental processes, plays a significant role in the Earth's biodiversity, climate, and water cycles. Because of its sparse human population and minimal industrial activities, the Qinghai-Tibet Plateau is often regarded as being relatively pristine [3]. However, human activities on the plateau over the last half-century have begun to alter land uses, resulting in a significant impact on local and regional climate [4]. In addition, pollutants directly introduced by human activities or indirectly inputted via atmospheric precipitation have started to pose a threat to the ecological communities in this region.

Conventional contaminants that have been found in this region include heavy metals [5,6], polycyclic aromatic hydrocarbons and organochlorine pesticides (OCPs) [7,8], and polychlorinated biphenyls (PCBs) [9]. Several newly emerging compounds, such as polyfluoroalkyl substances and hexabromocyclododecanes, have also been widely detected in some wild fish on the Qinghai-Tibetan Plateau, including *Oxygymnocypris stewartii, Schizopygopsis younghusbandi, Schizothorax macropogon, Schizothorax o'connori, Schizothorax waltoni, Gymoncypris waddellii, Gymoncypris przewalskii*, and *Racoma tibetanus* [3]. Antibiotic resistance genes (ARGs) have been recognized as emerging pollutants since 2006 [10]. ARGs are ancient and not synthesized by human, but could be accelerated by the selection pressure of various contaminants [11], such as antibiotics [12], heavy metals [13,14], even polycyclic aromatic hydrocarbons [15,16].

The wetlands of the Qinghai-Tibetan Plateau are located in the transitional zones between aquatic and terrestrial ecosystems [2]. Riverine, lacustrine, and palustrine wetlands are three of the most important types of wetlands in the region. Riverine wetlands are those associated with channels (e.g. river, creek or waterway) and streamside vegetation. Lacustrine wetlands are closely associated with lakes, which are dominated by open water with fringing vegetation. Palustrine wetlands are vegetated, non-riverine or non-channel systems, including billabongs, swamps, bogs, springs, and soaks, and have more than 30% emergent vegetation [17,18]. ARGs in wetlands can be enriched by the selection pressures of pollutants transported by rivers, streams, or runoff from terrestrial ecosystems. Aquatic ecosystems are regarded as an ideal setting for propagation and accumulation of ARGs [19]. In most previous studies on ARG propagation, artificially constructed wetlands, which were designed to remove the ARGs from sewage, have been the focus [20–25]. Few studies have assessed ARGs in natural wetlands. Furthermore, studies that have looked at ARGs in more natural wetlands or lakes have been performed at a local scale, often only one or two wetlands or lakes [26–29].

In this study, 32 wetlands across the Qinghai-Tibetan Plateau were studied. The distance between sites reached upwards of 2463 km (Fig. 1). Moreover, the environmental settings of these 32 wetlands were highly heterogeneous. The average temperature for these 32 wetlands, for example, ranged from -3.52 °C to 17.62 °C. Given these novel conditions, we first set out to determine if ARGs could be detected in these wetlands and if they displayed spatial heterogeneity. Secondly, because Qinghai-Tibetan Plateau is regarded as relatively pristine, we examined the influence of microbial community and environmental factors other than anthropogenic pollution on shaping the diversity and biogeography of ARGs in the wetlands. Finally, we assessed the relationship between climate factors (e.g., soil moisture and temperature) and ARG diversity and abundance to understand the effects of climate change on ARGs. This study not only gives a comprehensive profile of ARGs in different wetlands across the Qinghai-Tibetan Plateau but also identifies the key factors in shaping ARG diversity and geographic distribution.

2. Material and methods

2.1. Sampling sites and soils samples collection

In July 2014, surface (0–10 cm) soil samples were taken from 7 riverine wetlands, 19 lacustrine (lake margin) wetlands and 6 palustrine wetlands across the Qinghai-Tibetan Plateau (Fig. 1 and Table S1). These isolated wetlands were non-randomly selected but largely chosen based on site accessibility. At each wetland site, five soil subsamples within a 1-m^2 plot were collected randomly using a soil corer (3 cm diameter) and mixed together for DNA extraction and soil properties analysis. For each site, approximately 10 g of soils were collected in a centrifuge tube and immediately frozen in liquid N_2. In addition, for determining soil physical-chemical properties, 300–400 g of fresh soils were sealed in sterilized plastic bags and stored at 5 °C in a portable refrigerator.

2.2. Analysis of environmental factors in soil samples

The altitude, longitude and latitude of each sampling site were recorded with a global positioning system (Unistrong Co., Ltd, Beijing, China). The longitudes and latitudes were then imported into ArcGIS 9.3 (ESRI, California, USA) to extract the annual average temperature (°C) and precipitation (mm) from the WorldClim version 2.0 dataset (http://www.worldclim.org) at a spatial resolution of 30 s (~1 km^2).

In the field, the water depth of sampling sites was determined with a tape measure and the water conductivity was measured with a HI991301 water quality meter (Hanna Instruments, Padova, Italy). The concentrations of total carbon (TC), total organic carbon (TOC), total nitrogen (TN), and total phosphorus (TP) in the water samples were determined according to the methods described in our previous study [30].

For soil physical-chemical properties, soil temperature was determined on site by inserting a temperature probe into the 5-cm soil layer of each wetland site. In the laboratory, soil pH and conductivity were measured based on a 1:5 (v/v) soil to water ratio using a HI991301 water quality meter (Hanna Instruments, Padova, Italy). Soil moisture, soil TC, ammonia (NH_4^+) and nitrate (NO_3–) concentrations were analyzed using procedures outlined in [30]. In addition, soil TP was measured by an acid digest followed by a colorimetric assay using the molybdenum blue method [31]. Detail information of environmental factors for each sample was presented in the supplementary materials (Table S1).

2.3. DNA extraction and high-throughput quantitative polymerase chain reaction

The soil samples in the 32 wetlands were lyophilized and ground. DNA was then extracted from the fine sample with FastDNA Spin Kit for Soil (QBiogene, Carlsbad, CA) according to the protocol provided by the manufacturer, then purified by Geneclean Spin Kit (QBiogene, Carlsbad, CA) to minimize PCR inhibition. The quality and quantality of extracted DNA were determined by 1.5% agarose gel electrophoresis and spectrophotometer analysis (NanoDrop ND-2000c, Thermo).

A total of 118 genes were determined by high-throughput quantitative polymerase chain reaction (HT-qPCR) (Wcgene Biotech, Shanghai, China), including 16S rRNA, six transposases (*tnpA-02*, *tnpA-03*, *tnpA-04*, *tnpA-05*, *tnpA-06* and *tnpA-07*), two integron-integrase genes (*intI1* and *intI2*) and 109 targeted resistant genes conferring resistance to all major class of antibiotics (Table S2). The primer sets of tested genes and detail information about the process of raw data could also found from the previous publication [32].

2.4. 16S rRNA gene sequence analysis

The genomic DNA extracts served as a template for the PCR amplification of the V2-V4 region of 16S rRNA using the primer set 338 F/806R (5′-ACTCCTACGGGAGGCAGCAG-3′ and 5′- GGACTACHVGGGTWTCTAAT-3′) [33,34]. The amplified DNA was subjected to agarose gel

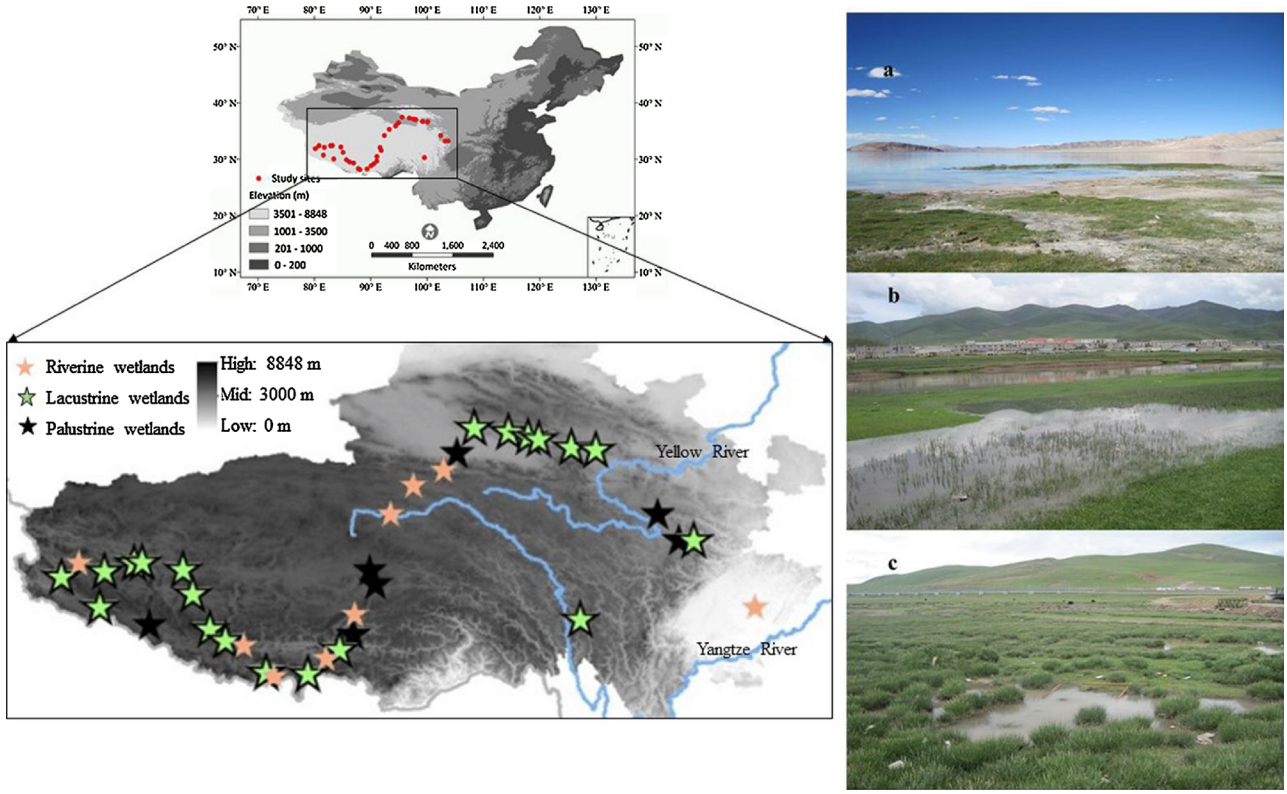

Fig. 1. Locations and typical environment of the studied wetlands on the Qinghai-Tibetan Plateau (lacustrine (a), riverine (b), and palustrine (c) wetlands).

electrophoresis and purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). A mixture of the amplicons was then used for sequencing on an Illumina MiSeq platform according to the standard protocols at Novogene Bioinformatics Technology Co., Ltd (Beijing, China). The 16S rRNA gene sequence data were processed and analyzed using QIIME version 1.4 as previously outlined [35]. In brief, all sequence analyses were carried out using the QIIME analysis pipeline. Operational taxonomic units (OTUs) were picked at 97% sequence identity and taxonomic identity using UPARSE pipeline [36].

2.5. Statistical and network analysis

Bar charts were created in Microsoft Excel version 2016. Pearson analysis was performed using Sigmaplot 12.5 (Systat Software, Inc., San Jose, CA, USA). Heatmaps were generated using R 3.4.1 (R Foundation for Statistical Computing, Vienna, Austria) with the pheatmap package [37] or heatmap.2 function within the gplots package [38]. Canonical correlation analysis was used to understand the effect of bacterial community, environmental factors, and altitude on the ARG profile in wetlands across the Qinghai-Tibetan Plateau, which was performed by R3.4.1 with the vegan package [39]. Spearman analysis was carried out using R3.4.1 with the Hmisc package [40] and the results were further analyzed to generate the network analysis using Gephi software v. 0.9.1 (Gephi, WebAtlas, France) [41]. Bray-Curtis distance values of ARGs and geographical distances were analyzed using R3.4.1 with the ecodist package [42].

3. Results

3.1. Antibiotic resistance genes in soils of different types of wetlands

Soil samples were collected from 7 riverine wetlands, 19 lacustrine wetlands, and 6 palustrine wetlands across the Qinghai-Tibetan Plateau in 2014 (Fig.1 and Supplementary Table S1). Among the 109 ARGs tested, 103 were recovered in these wetlands soil samples. In general, between 58–80 ARGs were detected in each sample from the riverine wetlands, 47–76 ARGs from each sample from the lacustrine wetlands, and 43–79 ARGs from each sample from the palustrine wetlands, each sample contain nearly every major classes of antibiotics used in human and animals (Fig. 2a). The diversity of ARGs detected among the 32 wetlands was compositionally similar; FCAs accounted for 21.93%, followed by β-lactamase resistance genes (16.11%), MLSBs (15.59%), aminoglycosides (15.57%), and tetracycline resistance genes (12.96%). All major ARG resistance mechanisms were detected in the wetland soils, and there was no significant difference in their ratios (Fig. 2b). Antibiotic deactivation for the resistance mechanisms of ARGs was the most commonly detected (38.22%), followed by efflux pumps (35.07%) and cellular protection (19.74%) (Fig. 2b).

The abundances of ARGs detected in the 32 wetlands are shown in Fig. 3. ARG abundances ranged from 1.80×10^5 to 1.35×10^7 copies per gram of soils, with an average value of 3.03×10^6 copies per gram of soils. The relative abundances of ARGs in the wetlands are between 8.08×10^{-3} and 3.19×10^{-1} with an average value of 5.23×10^{-2} resistance genes per 16S rRNA. There was no significant difference in the absolute abundances of total ARGs in the various types of wetlands via one-way analysis of variance ($p > 0.05$, Fig. S1). Our non-metric multidimensional scaling (NMDS) analysis indicates that there is no significant difference in the composition of ARG abundance in soil samples from the riverine, lacustrine and palustrine wetlands (Fig. S2). Generally, the abundances of FCA and β-lactamase resistance genes accounted for the highest proportion of ARG abundance, with average values of 38.29% and 36.74%, respectively (Fig. S3). The abundance of resistance mechanism of deactivation accounted for 48.49% of total ARGs in soils from wetlands on Qinghai-Tibetan Plateau, followed by the abundance of efflux (34.20%). ARG abundances and compositions in the 32 wetland soils varied from one to another (Fig. S3). Of the 103 ARGs detected, 17 were recovered in all wetland soils. These ARGs represent all the main resistance mechanisms (deactivation, efflux, protection, and unknown), including 7 FCA resistance genes and 4 others ARGs, 3 β-lactamase resistance genes, 1 MLSB resistance gene, 1 tetracycline gene, 1 vancomycin gene. *blaTEM*, *mexF*, *mphA-01*, *oprJ*, *oprD*, *blaSFO*, *fox5*, *mepA*, *mphA-02*, *acrA-04*, *vanC-03*, *qacH-01*, *qnrS* and *pica*, which conferred

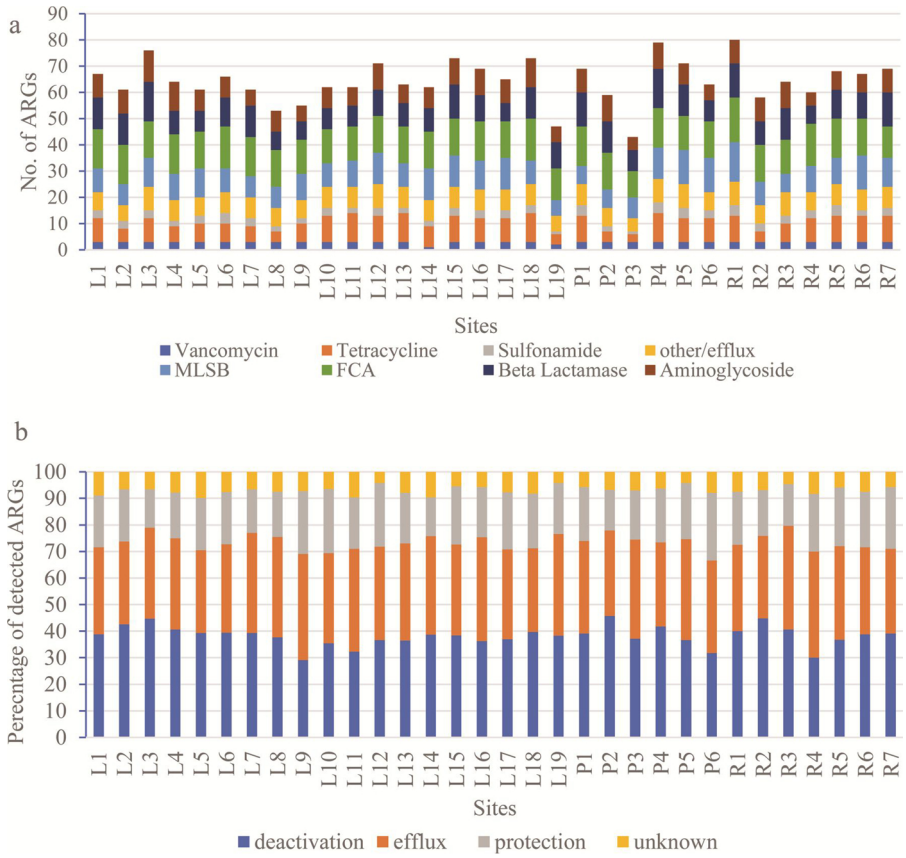

Fig. 2. The number of detected ARGs (a) and their percentage for resistance mechanisms (b) (FCA, fluroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance).

resistance to β-lactamase, FCA, MLSB, aminoglycoside, vancomycin and other antibiotics were consistently detected at high abundances, with average values of more than 8×10^4 copies per gram of soil (Fig. 3).

3.2. Geographical analysis of ARGs variation

ARG abundance and diversity were recorded in soil samples from 32 wetlands, spread out across 2463 km. The Bray-Curtis distance values of these ARGs and geographical distances were not normally distributed and a Spearman correlation analysis was used to discover the effects of geographical distances on the ARG variation. Based on these measures, geographical distance did not influence the composition of ARG profiles (Spearman correlation coefficient = 0.04, $p = 0.339$). Similarly, altitude and ARG abundances were not normally distributed so again a Spearman correlation analysis was used. In general, the altitude had a negative effect on the total abundance of ARGs (Spearman correlation coefficient $= -0.483$, $p < 0.01$). Altitude had a negative effect on the abundances of ARGs conferring resistance to vancomycin ($r = -0.633$), sulfonamide ($r = -0.502$), MLSB ($r = -0.484$), β-lactamase ($r = -0.487$) and FCA ($r = -0.394$) at $p < 0.05$ level. However, no significant correlation was found between altitude and the abundance of ARGs conferring resistance to tetracycline, aminoglycoside and other/efflux.

3.3. Mobile genetic elements (MGEs) in soils of different types of wetlands

Six transposase genes (*tnpA-02*, *tnpA-03*, *tnpA-04*, *tnpA-05*, *tnpA-06* and *tnpA-07*) and class 1 and 2 integron-intergrase genes were selected to investigate the transfer or propagation of ARGs in soils of wetlands across the Qinghai-Tibetan Plateau. These mobile genetic elements in wetlands ranged from 3.13×10^3 to 1.02×10^6 copies per gram of soil. The copy number of transposase genes and integron-intergrase genes in wetlands were in the range of 8.50×10^2 –9.92×10^5 and 2.28×10^3 – 3.43×10^5 copies per gram of soil, respectively. The absolute abundances of the ARGs were significantly correlated with the abundances of integrons at $p < 0.01$ level, but were not significantly correlated with the abundances of transposases ($p > 0.05$). The relative abundance of ARGs significantly correlated with the relative abundance of integrons (Pearson's $r = 0.415$) and transposases (Pearson's $r = 0.355$) at $p < 0.05$ level (Fig. 4).

The co-occurrence of ARGs and MGEs was explored with a Spearman correlation analysis using Gephi software, which showed the clusters of ARG and MGEs in the network with Spearman's correlation coefficients over 0.60 and $p < 0.01$ (Fig. 5). Class 1 integrons played an important role in the propagation of different kinds of ARGs [11,43]. Twenty-one ARGs provided resistance to seven kinds of antibiotics (vancomycin, tetracycline, sulfonamide, other/efflux, MLSB, FCA, and beta-lactamase) were clustered with Class 1 integron (Table S3). Seven modules were recovered with a modularity analysis, with two large modules detected. Different kinds of ARGs were clustered in each module, indicating dense connections between different classes of ARGs within each module. *VanC-03* and *tet(34)* were the hub genes for the two largest modules; these could be used as indicators for ARG co-occurrence in the two modules.

3.4. Bacterial community in soils of wetlands

The absolute bacterial abundances in the wetland soils from across the Qinghai-Tibetan Plateau ranged from 2.23×10^6 - 5.36×10^8 copies per gram of soil. The absolute abundances of these bacteria exhibited a strong positive relationship with the total ARG abundance (Pearson's $r = 0.762$, $p < 0.01$). The abundances of ARGs for each major class of antibiotics was also strongly correlated ($p < 0.01$) with the bacterial abundance, except for aminoglycoside and tetracycline

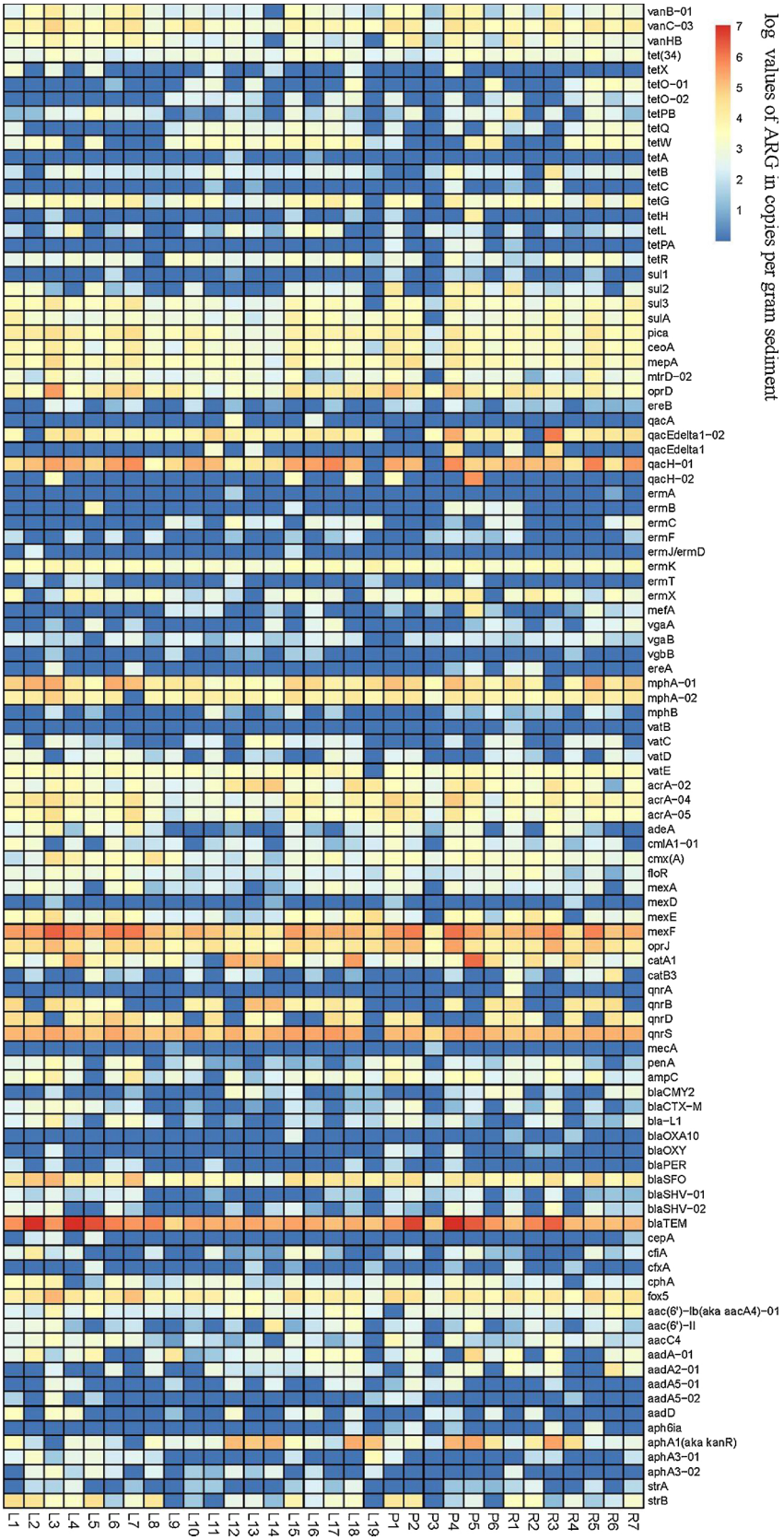

Fig. 3. The log values of absolute abundances of antibiotic resistance genes in riverine (R), lacustrine (L) and palustrine (P) wetlands.

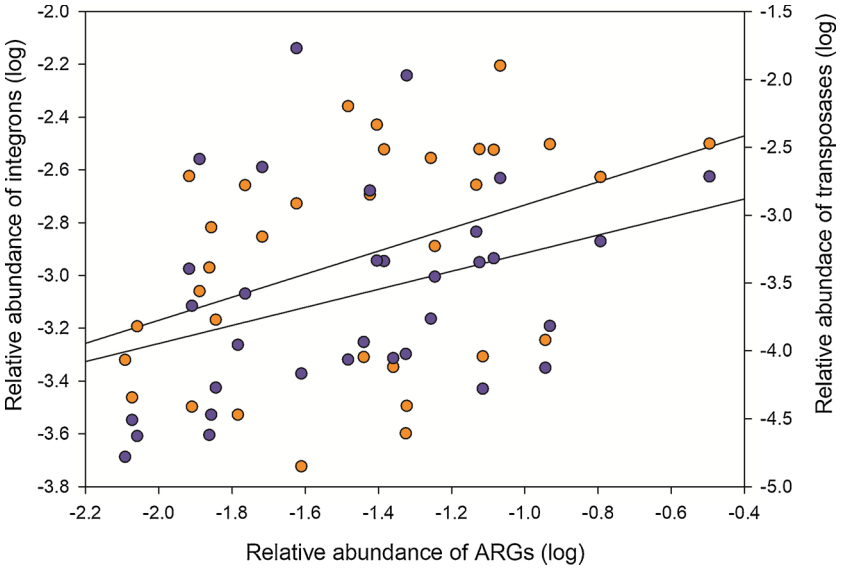

Fig. 4. The Pearson correlation relationship between the relative abundances of ARGs and mobile genetic elements (brown circle for integrons, blue circles for transposases; Integron-intergrase genes, Pearson's r = 0.415, $p < 0.05$; Transposase, Pearson's r = 0.355, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

resistance genes (Table S4). Bacterial abundance was strongly correlated with the integron abundance (Pearson's r = 0.738, $p < 0.01$), but no significant correlation was found with transposases (Pearson's r = 0.314, $p = 0.075$). These results indicate that bacterial abundance may directly influence ARG and integron abundance in wetlands across the Qinghai-Tibetan Plateau.

The diversity and richness of bacterial communities were assessed with 16S rRNA sequencing and a high degree of coverage (Good's coverage of > 94%) was found (Table S5). The OTU number of 16S rRNA varied from site to site, ranging from 2520 to 6279. Diversity estimates based on a Shannon index of the 32 wetlands ranged from 7.64 to 10.44. The compositions of bacterial communities among the 32 wetlands were not similar at the phylum level (Fig. 6). *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Chloroflexi* and *Firmicutes* accounted for more than 75% of bacterial community structure in the wetlands. *Proteobacteria* was the most abundant phylum in the soils, comprising between 35% and 62% of the bacterial communities. Co-occurrence pattern of ARG and bacterial community at the genus level revealed through a network analysis showed that most bacterial taxa were clustered together without ARGs (Fig. S4). Four ARGs were strongly correlated with their possible hosts (Table S6). Based on these findings, the co-occurrence between ARGs and bacterial communities does not appear to be an efficient method to determine the composition of antibiotic resistant bacteria in the wetlands.

3.5. Correlation between ARG abundances with environmental factors

The relationship between environmental variables and ARG abundance was examined with heatmaps and Spearman correlation analysis (Fig. 7). The environmental variables could be divided into two groups: Group I included water depth, average temperature, soil ammonium, soil moisture, soil temperature, and soil total carbon, whereas Group II included soil pH, total phosphorus in water, soil nitrate, precipitation, soil total phosphorus, total nitrogen and total organic carbon in water, water conductivity, and soil conductivity. Soil moisture and soil total carbon were positively correlated ($p < 0.05$) with five classes of ARGs conferring resistance to sulfonamide, vancomycin, MLSB, others, and FCA. Average temperature, soil ammonium, and soil temperature were positively correlated ($p < 0.05$) with 2–3 class of ARGs. The environmental variables in group II had no significant correlations with any class of ARGs. Correlations between individual ARG abundance and environmental factors are shown in the network ($p < 0.05$) (Fig. S5). Five modules were found based on the modularity analysis. Soil moisture was the hub in the largest module with soil ammonium and soil TC. The 32 ARGs conferring resistance to all major kinds of antibiotics were found to have a positive correlation with soil moisture ($p < 0.05$). Soil temperature was the hub of the second largest module. Notably, soil conductivity and conductivity were the hub of the fourth module, which had a negative influence on specific individual ARG abundance. These results seem to confirm that soil physiochemical characteristics had a significant influence on the ARG abundance.

Canonical correlation analysis (CCA) was used to use to understand the effect of bacterial community, environmental factors, and altitude on the ARG distribution. It was found that bacterial community composition could fully explain the variation of ARGs in the wetlands (Table S7). Environmental factors and spatial variables showed no contribution when analyzed together with bacterial community composition. Therefore, environmental factors and altitude were analyzed together to investigate the effect of abiotic factors on the ARG variation in wetlands across the Qinghai-Tibetan Plateau (Fig. S6). Environmental factors accounted for 65.03% of the variance of ARG distribution, followed by altitude (2.95%), and their interaction (0.88%). Bacterial community composition, followed closely by environmental factors, was the main drivers in shaping the diversity and biogeography of ARGs in wetlands across the Qinghai-Tibetan Plateau.

4. Discussion

ARGs were detected in levels ranging from 10^5 to 10^7 copies per gram of soil in the wetlands of the Qinghai-Tibetan Plateau, levels slightly lower than ARG abundance ($\sim 8.8 \times 10^5$–1.8×10^8 copies per gram of sediment) in the estuaries from China [32]. Metagenomics showed that the ARGs in these relatively pristine Tibet environments were compositionally different than those found in sewage treatment plants in Hong Kong and husbandry in Zhuhai, China [44]. The average relative abundance of ARGs in these wetlands was 13 times lower than those in coastal estuaries [32] and in the eastern urban Tiaoxi River with rapid urbanization and increased human activities [45]. Antibiotic deactivation and efflux pumps were the two most dominant resistance mechanisms in wetlands across the Qinghai-Tibet Plateau. Similar results were also observed in Tiaoxi River [45] and Wen-Rui Tang River [46], estuaries from across China [32], and soils with direct application of sewage sludge compost [47]. These results indicated that antibiotic deactivation and efflux pumps may be the two most dominant resistance in a diverse array of environments, including both pristine and polluted environments. FCA and β-lactamase resistance genes comprised a large proportion of the ARG abundance in these wetlands. With regards to individual ARGs, *mphA, oprJ* and *oprD* exhibited high abundance compared with other tested ARGs, both in wetlands studied here and in the coastal

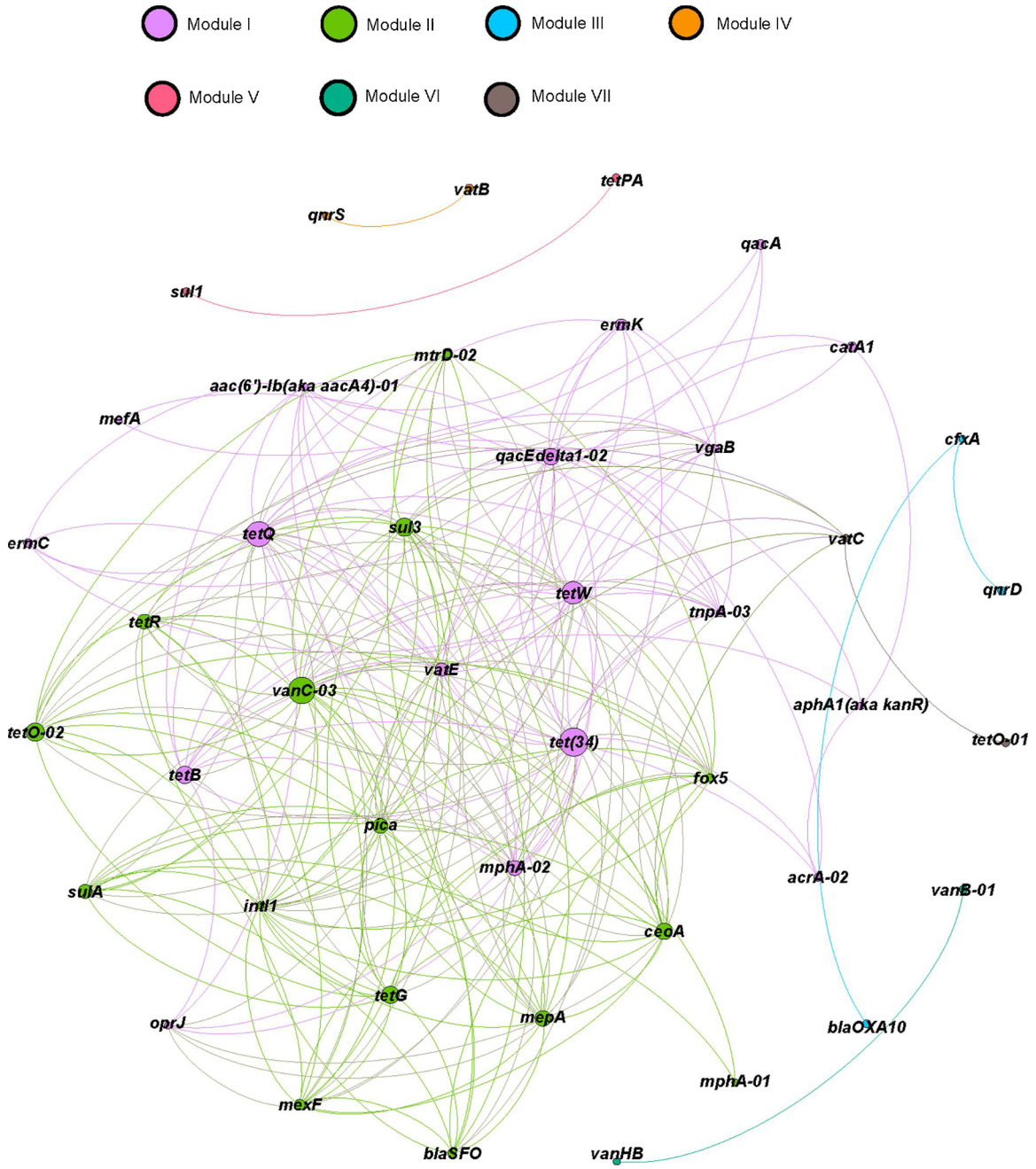

Fig. 5. Network analysis of co-occurrence patterns among antibiotic resistance genes, transposases and the class 1 integron-integrase gene *(intI1)* based on the Spearman's r > 0.60 and $p < 0.01$.

estuaries [32]. Although the coastal estuaries were influenced by pollutants released by rivers and waterway and acting as natural filtering points for pollutants [32], the Qinghai-Tibetan Plateau wetlands could be regarded as relatively pristine sites. This seems to indicate that the levels of *mphA, oprJ* and *oprD* may be less influenced by human activities. Wetlands from the Qinghai-Tibetan Plateau are important freshwater sources for Tibet's drinking water and food sources for local people [48], and a shared antibiotic resistome has been observed between soil bacteria and human pathogens [49]. The ARGs in the wetlands could pose a serious threat to human health [11].

The levels of *sul1* in the wetlands (not detected – 2.2×10^{-6} copies per 16S rRNA) were comparable to those detected in polar marine sediment samples (2.45×10^{-7}–1.71×10^{-6} copies per 16S rRNA), another relatively pristine site [50]. The *sul* genes in the 32 wetlands in the Qinghai-Tibetan Plateau were approximately 2 to 5 orders of magnitude lower than those from sediments collected from 15 lakes along the middle and lower reaches of Yangtze River (10^{-4} – 10^{-1} copies per 16S rRNA) [51]. Vancomycin resistance genes are relatively ancient genes in natural environments [52] and were detected in most wetlands across the Qinghai-Tibetan Plateau. The abundance of vancomycin resistance genes in wetlands ranged to 8.56×10^{4} copies per gram of sediment, which is two orders of magnitude lower than those detected in coastal estuaries [32].

The ARG diversity and abundance in the wetlands suggests that the Qinghai-Tibetan Plateau is an important gene pool for ARGs, even it

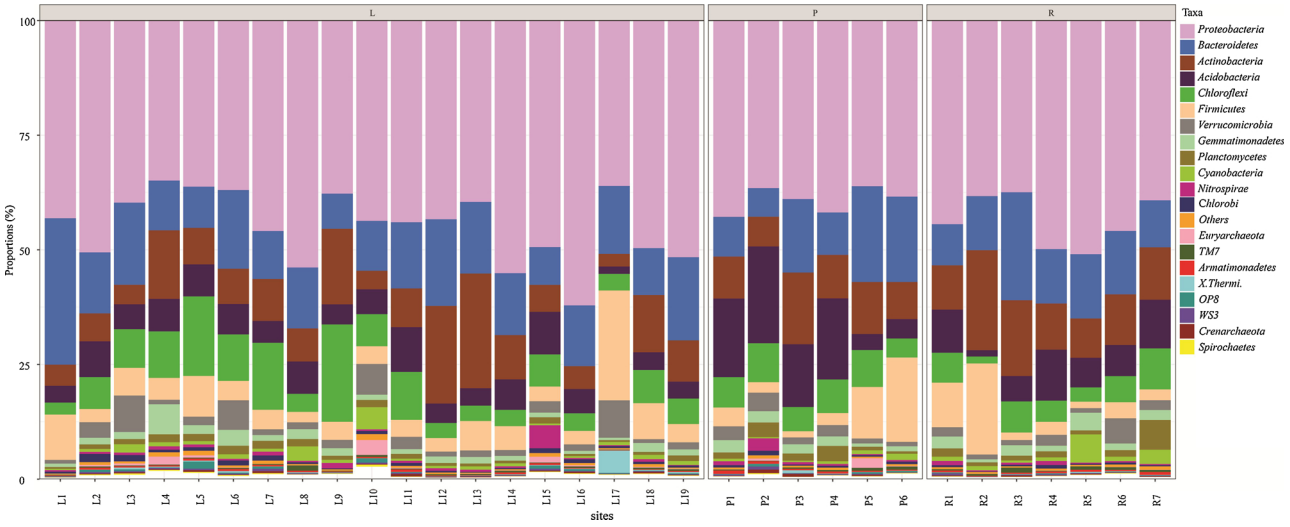

Fig. 6. The compositions of bacterial community at phylum level in soils from in riverine (R), lacustrine (L) and palustrine (P) wetlands.

was less influenced by anthropogenic factors [53]. With its thin air and heavy permafrost, the high-altitude region of the Qinghai-Tibetan Plateau is one of the least populated regions of China, especially the mid-western parts. Altitude had a strong negative correlation with the total number of ARGs, indicating that high altitude with low intensity of human activities usually had the low abundance of ARGs. The clinical class 1 integron-integrase gene was detected in all wetlands across the Qinghai-Tibetan Plateau. The *intI1* was an important mobile genetic element to acquire and disseminate ARGs. Altitude was strongly negatively correlated with *intI1* abundance (Spearman's $r = -0.499$, $p < 0.01$). This confirms that high altitude contributes to low abundance of ARGs and *intI1*, which could be also regarded as proxy for intensity of human actives or anthropogenic pollution [54]. *intI1* was initially discovered to acquire a gene for efflux pump (*qacE*Δ) that is only originated from pathogens and bacteria derived from humans or domestic animals [55]. *qacE*Δ*1-02* was detected in all wetlands across the Qinghai-Tibetan Plateau and had a significantly positive correlation with *intI1*. *intI1* also played an important role in the acquisition and spread of sulfonamide, tetracycline, MLSB, and β-Lactamase resistance genes. These results show that, among the 109 ARGs tested, 21 genes could be identified as being derived from anthropogenic pollution and were propagated by the *intI1*. The absolute abundances of *intI1* in the wetlands of Qinghai-Tibetan Plateau were lower than those in coastal estuaries [32]. Additionally, the relative abundance of *intI1* was also

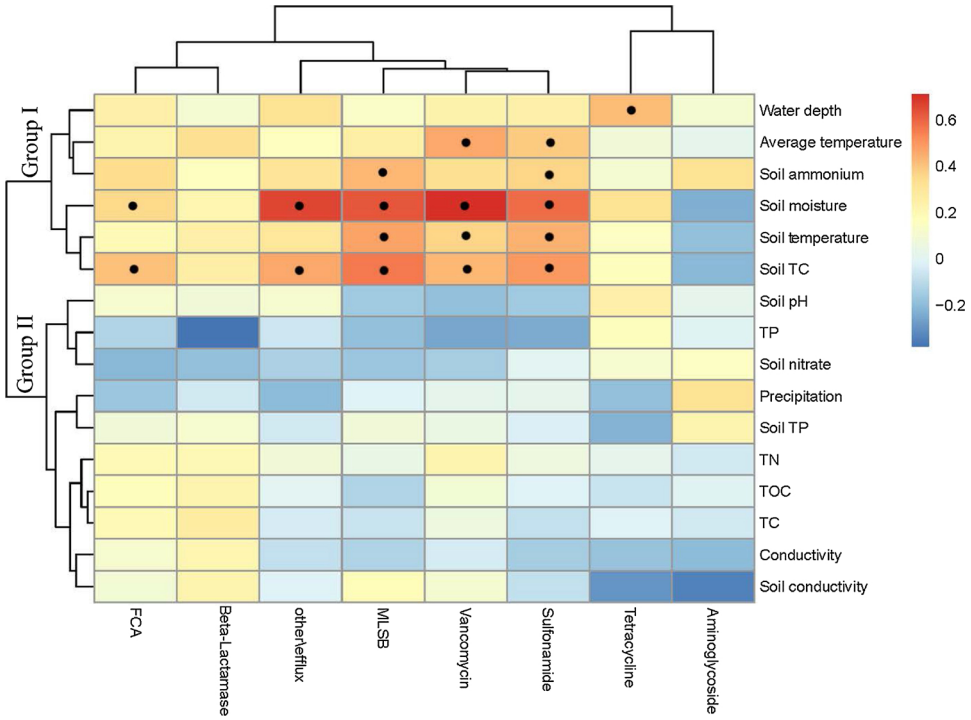

Fig. 7. The correlation relationship between the ARG abundances and environmental factors based on Spearman analysis (dot means $p < 0.05$). TP: total phosphorus; TC: total carbon; TN: total nitrogen; TOC: total organic carbon.

lower than those in lakes sediment along the middle and lower reaches of Yangtze River [51]. A meta-analysis demonstrated that *intI1* were commonly detected over 1×10^6 copies per gram diverse environmental samples (rivers, lakes, and wastewater) [43], which were higher than the level recovered in this study. This indicates that the transfer rate of ARGs between bacteria in the Qinghai-Tibetan Plateau via *intI1* may be lower because of the reduced propagation of ARGs in this region. Similar findings were also recovered in assessments of ARG and MGE relationships in the Tibetan environment [44]. Collectively, these findings suggest that the risk of *intI1* in wetlands across the Qinghai-Tibet Plateau may be at a low level.

The bacterial communities present in these relatively pristine wetlands of the Qinghai-Tibetan Plateau may also explain some of the ARG profiles and distributions observed in this study. Similar results were also observed in American agricultural and grassland soils where bacterial phylogeny structured soil resistomes were found [56]. In contrast, anthropogenic factors other than bacterial communities were suggested to be the main drivers influencing the ARG profiles and distribution in the coastal estuaries [32]. The bacterial communities seen in the 32 wetland sites across the Qinghai-Tibetan Plateau displayed notable variations from each other, whereas the bacterial composition of the 18 coastal estuaries was similar [32]. The proportion of build-up land use, an indicator of human intensity, also played an important role in the abundance and distribution of ARGs in the lake sediments from the Yangtze River [51]. Human activity is also thought to have impacted the abundance of ARGs in 21 Swiss lakes [57]. In the Qinghai-Tibetan Plateau, however, the intensity of human activities is significantly lower than these other environments, principally through protection by the local government to reduce human interference.

Co-occurrence of ARGs and bacterial taxa using network analysis based on Spearman correlation analysis was used to identify the potential host of ARGs [32,58]. In lakes and reservoirs across east and northeast China, *Chitinophagaceae* bacterial OTUs had the most connections with the ARGs, followed by *Planctomycetaceae* and *Sphingomonadaceae* [58]. *Brevundimonas* and *Methylobacterium* were suggested to be potential hosts for *strA* and *strB* in the finished water after treatment [59]. In the current study, *Cytophagia*, *Oscillatoriophycideae*, *Gammaproteobacteria* and *Methanomicrobia* are thought to be potential hosts of ARGs in wetlands; however, metagenomic sequencing or isolation of antibiotic resistant strains are still need to determine the definitive hosts [32].

Environmental factors and spatial distances are important drivers shaping microbial communities and functional genes [60–63]. In this study, we found that geographical distance had no statistical relationship with ARG profile and distribution, indicating that spatial distance between these wetlands is not an important factor in shaping ARG diversity. Conversely, soil conditions and, to a lesser extent, altitude are factors that influenced ARG profiles in the wetlands. In particular, altitude influenced the abundance of ARGs, but not their diversity. Soil moisture, temperature, and conductivity were also determined to be important factors for both ARG diversity and abundance; notably, soil moisture and temperature were also important factors on microbial diversity (Fig. S7). Recent changes in soil temperature and moisture in the Qinghai-Tibetan Plateau, caused by a warming climate and fluctuations in precipitation, have not only had direct changes on soil microbes habitats (e.g. soil nitrogen and carbon) [64] but have also altered the diversity and composition of soil microbial communities [65–67]. Dynamic moisture changes in soils have been shown to accelerate the susceptibility of the soil microbial community to antibiotics [68]. Soil moisture in the studied wetlands was also positively correlated with ARG abundance, which may indicate that soil moisture is not only a factor for microbial communities but also accelerate the transfer of ARGs among the bacteria community.

Temperature has previously been revealed to positively correlated with ARG abundance in engineered systems [69]. Similarly, in our study, we found that temperature played an important role in ARG abundance in these natural wetlands. The warming climate trend in the Qinghai-Tibetan will very likely lead to increased soil temperature and moisture which will alter the microbial community composition and increase the background levels of ARGs in the wetlands. Conductivity, an indication of the total dissolved metal concentration, had a negative effect on the sedimentary archaeal *amoA* gene abundance in Qinghai Lake [70] and helped shape the denitrifier community in wetland soils across the Qinghai-Tibetan Plateau (data not published). To the best of our knowledge, no other study has been carried out on the effects of conductivity on ARG diversity and abundance in wetlands. Conductivity had a negative influence on specific individual ARG abundance in this study.

At this time, we cautiously proposed that climate change and anthropogenic factors indirectly influence the ARG profile and distribution via the bacterial community. If human activities in this region substantially increase, especially in the eastern Qinghai-Tibetan Plateau, direct input of ARGs via anthropogenic pollution will become an important factor in shaping the ARG diversity, just as witnessed in the coastal estuaries in China [32]. Microbiome sequencing or isolation of antibiotic resistant bacteria in this region are needed to get a profile of antibiotic resistant bacteria and their ARGs and MGEs. Currently, low temperature and human interference have led to the low background levels and transfer rates of ARGs in the Qinghai-Tibetan Plateau wetlands; however, with climate warming and increased anthropogenic factors, studies on different scenarios are urgently needed to understand changes in ARG and antibiotic resistant bacteria.

Competing interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2018.09.002.

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